Neuron, Vol. 23, 385–397, February, 2000, Copyright ©2000 by Cell Press

A Novel Targeting Signal for Proximal Clustering of the Kv2.1 K^+ Channel in Hippocampal Neurons

Seung T. Lim, Dana E. Antonucci, Robert H. Scannevin,† and James S. Trimmer*
Department of Biochemistry and Cell Biology and Institute for Cell and Developmental Biology
State University of New York
Stony Brook, New York 11794

Summary

The discrete localization of ion channels is a critical determinant of neuronal excitability. We show here that the dendritic K^+ channels Kv2.1 and Kv2.2 were differentially targeted in cultured hippocampal neurons. Kv2.1 was found in high-density clusters on the soma and proximal dendrites, while Kv2.2 was uniformly distributed throughout the soma and dendrites. Chimeras revealed a proximal restriction and clustering domain on the cytoplasmic tail of Kv2.1. Truncations and internal deletions revealed a 26–amino acid targeting signal within which four residues were critical for localization. This signal is not related to other known sequences for neuronal and epithelial membrane protein targeting and represents a novel cytoplasmic signal responsible for proximal restriction and clustering.

Introduction

Neurons are highly differentiated cells which rely on discrete localization of specific neurotransmitter- and voltage-gated ion channels to generate and propagate electrical signals that underlie neuronal activity. Tremendous insights have been gained into components of the synapse and the protein–protein interactions critical to neurotransmitter receptor localization at these specialized membrane domains (Kim and Huganir, 1999; Sheng and Pak, 1999). While discrete localization of voltage-gated ion channels at precise sites in somal, dendritic, and axonal membranes is also critical to neuronal signaling, the underlying mechanisms are not well defined and remain an important and interesting problem.

Voltage-gated K^+ (Kv) channels, as key determinants of membrane excitability, contribute to the wide range of neuronal phenotypes observed in the mammalian nervous system (Pongs, 1999). Classically, Kv channel function was associated with axons (Hille, 1992); however, recent studies have defined a role for somal and dendritic Kv channels in regulating processing of synaptic signals and backpropagating action potentials (Hoffman et al., 1997). The mammalian Kv channel gene family encodes at least two dozen distinct α subunit polypeptides (Chandy and Gutman, 1995), which contain pore-forming and voltage-sensing transmembrane domains and assemble as homo- or heterotetramers to generate a wide variety of possible subunit combinations with distinct functional properties (Trimmer, 1998). The heterogeneity of neuronal Kv channel gene expression, together with discrete and highly variable targeting of different Kv channel types to restricted neuronal membrane domains, generates a multiplicity of Kv channel function, expression, and distribution that helps shape the diverse electrical phenotype of individual neurons.

The Kv2.1 K^+ channel is thus somewhat atypical in its widespread expression in mammalian brain (Trimmer, 1991; Hwang et al., 1993; Rhodes et al., 1995; Scannevin et al., 1996; Du et al., 1998). Previous studies have shown that Kv2.1 is a major component of delayed rectifier currents in rat hippocampal neurons (Murakoshi and Trimmer, 1999) and underlies the slowly deactivating current that is a critical determinant of discharge patterning in rat globus pallidus neurons (Baranauskas et al., 1999). Given its high-level expression in most brain neurons, Kv2.1 would be expected to be a major determinant of neuronal activity. While Kv2.1 has an extensive cellular expression in brain, its subcellular localization in neurons is discrete and highly restricted to large clusters on the soma and proximal dendrites (Trimmer, 1991; Hwang et al., 1993; Rhodes et al., 1995; Scannevin et al., 1996; Du et al., 1998; Murakoshi and Trimmer, 1999). Based on the distinctive localization of Kv2.1 among Kv channels, we would predict that Kv2.1 contains a unique proximal restriction and clustering (PRC) signal that directs these events in neurons.

We previously studied targeting of Kv2.1 and two C-terminal truncation mutants in Madin–Darby canine kidney (MDCK) polarized epithelial cells and found that the cytoplasmic tail of Kv2.1 was necessary for both polarized targeting and clustering (Scannevin et al., 1996). While epithelial cells and neurons share many common mechanisms of protein targeting (Dotti and Simons, 1990), several neuronal proteins expressed in epithelial cells have unexpected localizations (discussed in Stowell and Craig, 1999). In addition, it is unlikely that epithelial cells have a domain analogous to the proximal portion of dendrites. Thus, there remains a clear need to characterize the PRC signal in neurons. Here, we analyze subcellular targeting and clustering of Kv2.1 in cultured hippocampal neurons and define a cytoplasmic domain that acts as a PRC signal and that is a distinct targeting signal in polarized epithelial cells or neurons.

Results

Recombinant HA-Kv2.1 Is Appropriately Polarized and Clustered When Expressed in Hippocampal Cultures

Immunofluorescence staining of rat hippocampal neurons grown in culture for 14 days (14 days in vitro or 14

*To whom correspondence should be addressed (e-mail: james.trimmer@sunysb.edu).
† Present address: Howard Hughes Medical Institute, Johns Hopkins University, Department of Neuroscience, Baltimore, Maryland 21205.

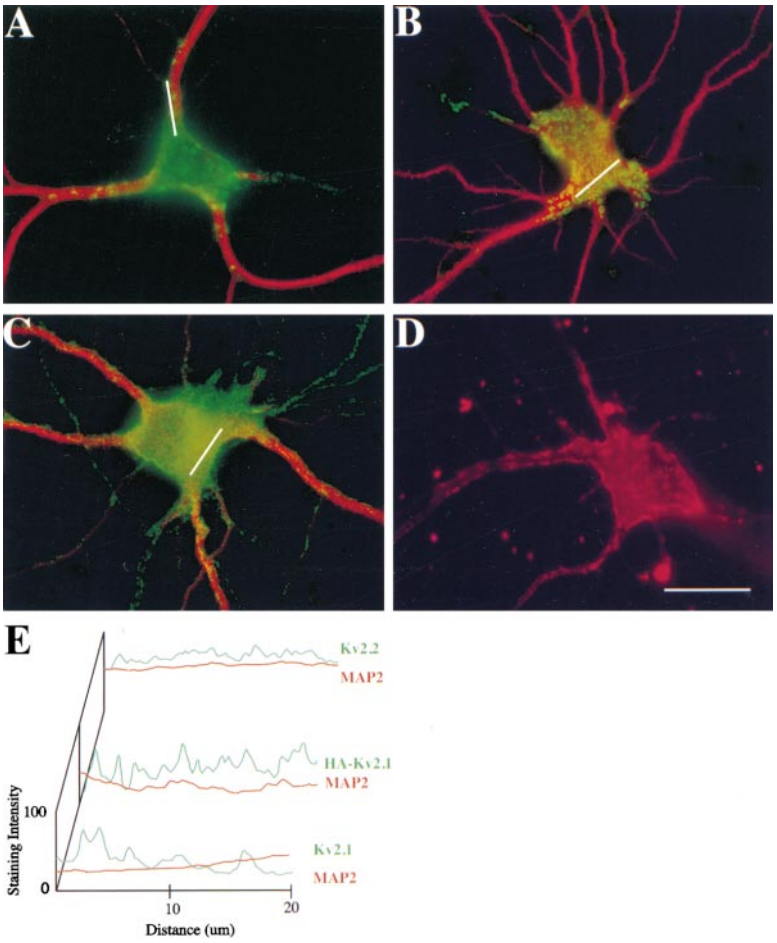

Figure 1. Recombinant Kv2.1 Has Appropriate Proximal Restriction and Clustering in Transfected Hippocampal Neurons

(A) Endogenous Kv2.1 expression in 14 DIV cultured hippocampal neurons. Endogenous Kv2.1 (green) was expressed in cell bodies and the proximal region of dendrites in high-density donut-shaped clusters. MAP2 staining (red) reveals the extent of the dendrites.

(B) The restricted and clustered distribution of recombinant Kv2.1 in hippocampal neurons. Neurons were transfected with HA-Kv2.1 at 7 DIV and immunostained 2 days later with 12CA5 for HA-Kv2.1 (green) and MAP2 (red). HA-Kv2.1 was localized to cell bodies and proximal dendrites but was not present in the distal portion of dendrites or axons. Note the prominent ring or donut shape of HA-Kv2.1 clusters.

(C) The distribution of recombinant Kv2.2, the most closely related Kv channel to Kv2.1. Neurons at 7 DIV were transfected with Kv2.2 cDNA and immunolabeled 2 days later with Kv2.2 antibody (green) and MAP2 (red). Staining of Kv2.2 is present uniformly throughout cell bodies and dendrites.

(D) Staining of cell surface HA-Kv2.1. HA-Kv2.1-transfected neurons were stained with the ectodomain-directed K39/25 anti-Kv2.1 monoclonal antibody in the absence of detergent permeabilization. Scale bar, 20 μm.

(E) Plots of relative staining intensity. The intensity of Kv2.1, Kv2.2, and MAP2 staining, was measured over a 20 μm segment on the neuronal cell body at the sites indicated by the white bars in each panel. Relative intensity values on the y axis range from a low 0 to a maximal value of 100 and are plotted against distance (in μm) on the x axis.

DIV) demonstrated a highly ordered pattern of endogenous Kv2.1 localization (green) restricted to cell bodies and very proximal portions of dendrites; MAP2 staining (red) reveals the extent of dendrites (Figure 1A). Kv2.1 staining was absent from more distal portions of dendrites and axons (Figure 1A). Kv2.1 staining in high-density patches on the soma and on proximal portions of dendrites in these cultures is similar to that in rat brain neurons (Trimmer, 1991; Hwang et al., 1993; Rhodes et al., 1995; Scannevin et al., 1996; Du et al., 1998) and in more mature cultures of hippocampal neurons (Murakoshi and Trimmer, 1999). The size of clusters ranged from 1 to 4.5 μm, with an average diameter of 3.24 ± 0.85 μm (n = 122). Kv2.1 clusters were donut- or ring-shaped in appearance, where the thickness of the ring was 1.42 ± 0.28 μm (n = 30). We used this information to define the following criteria for Kv.2.1 clustering that was used in the experiments. "Clustered" staining was defined as bright, ring-shaped foci, 1–5 μm in diameter, with much lower or undetectable staining between clusters. "Uniform" staining was defined as staining patterns distinct from either these clusters or any other identifiable restricted subcellular compartmentalization (e.g., to synapses).

Our strategy to elucidate mechanisms underlying neuronal protein targeting and clustering was based on expressing influenza hemagglutinin (HA) epitope–tagged wild-type and mutant Kv2.1 isoforms by transient transfection in cultured hippocampal neurons. That introduced HA-Kv2.1 in transfected neurons had the same localization as endogenous Kv2.1 was a necessary criterion for embarking on a structure-function analysis of the PRC signal on Kv2.1. We transiently transfected hippocampal neurons at 7 DIV with HA-Kv2.1 cDNA and stained with 12CA5 anti-HA monoclonal antibody to distinguish introduced HA-Kv2.1 from endogenous Kv2.1. Recombinant HA-Kv2.1 had a localization pattern identical to endogenous Kv2.1, being found in large clusters on cell bodies and proximal dendrites; again, MAP2 staining reveals the extent of dendrites (Figure 1B). Neither HA-Kv2.1 nor endogenous Kv2.1 was observed on the more distal portions of dendrites and axons. However, in cells at this relatively early stage of culture, the initial 60 μm of axons often contained MAP2 staining that was not seen in more mature cultures. In these cells Kv2.1 staining was also seen on axon initial segments (Figure 1B). In cells exhibiting MAP2 staining of axon initial segments, similar Kv2.1 staining was scored as lacking true axonal localization.

One feature of endogenous protein clustering that was more obvious upon overexpression of HA-Kv2.1 was the presence on the soma of large donut-shaped clusters of HA-Kv2.1 (Figure 1B). The thickness of the ring itself was 1.12 ± 0.19 μm (n = 30), and the average diameter

of the clusters was 3.54 ± 0.86 μm (n = 233). These values are similar to those obtained for clusters of endogenous Kv2.1 (see above), indicating that subcellular machinery for Kv2.1 clustering in these cultured hippocampal neurons operates similarly for endogenous and introduced Kv2.1 and that its capacity exceeds endogenous Kv2.1 expression levels. Interestingly, transfection experiments performed on neuron/astrocyte cocultures revealed that HA-Kv2.1 was not clustered in transfected astrocytes (data not shown).

Since expression of HA-Kv2.1 is driven by the cytomegalovirus (CMV) promoter, a concern was that overexpression could lead to saturation of Kv2.1 localization machinery and result in mislocalization of introduced Kv2.1, as was observed for targeting of metabotropic glutamate receptors expressed in hippocampal neurons from defective viral vectors (Stowell and Craig, 1999). We measured relative expression levels of endogenous Kv2.1 and HA-Kv2.1 proteins. At 48 hr after transfection, staining intensity of Kv2.1 (as measured by KC staining) in HA-Kv2.1-transfected neurons was approximately 9-fold higher (x = 8.9-fold, n = 10 cells) than that of endogenous Kv2.1 in untransfected cells. However, 80% of Kv2.1-transfected neurons exhibited proper localization and clustering up to 96 hr after transfection, and 93% exhibited HA-Kv2.1 localization at 48 hr posttransfection (Figure 5C), the time point used for the studies below. These data indicate that overexpression of HA-Kv2.1 by our transfection protocol does not alter its distribution or clustering relative to endogenous Kv2.1 and that overexpression does not saturate the PRC machinery.

We also verified that the bulk of introduced HA-tagged Kv2.1 was expressed on the cell surface by the fact that transfected (12CA5+) neurons had enhanced staining when an anti-Kv2.1 ectodomain-specific monoclonal antibody (K39/25) was applied to intact neurons (Figure 1D). For each of the HA-tagged Kv2.1 mutants described below, external K39/25 staining had the same distribution as 12CA5 staining (data not shown). Confocal microscopy provided independent support for this conclusion and revealed that the majority of large Kv2.1 clusters were on the bottom or basal surface of cultured neurons, at cell-substratum interfaces (data not shown).

Kv2.2, which is the other member of the mammalian *Shab*-related family of Kv channels and the protein most closely related to Kv2.1, was also expressed in hippocampal neurons by lipid-mediated transfection. The distribution of introduced Kv2.2 (green) was distinct from clustered Kv2.1, as it was expressed uniformly in the neuronal cell body and throughout all processes, and no ring-shaped clusters were observed (Figure 1C). Thus, expression of these two highly related recombinant Kv channels by transient transfection in cultured hippocampal neurons yields contrasting subcellular distributions that are similar to those observed for these channels in brain neurons (Hwang et al., 1993).

To visualize the extent of protein clustering in neurons, fluorescence intensity was examined across a 20 μm line along cell bodies or dendrites. Plotting staining intensity against distance thus graphically portrayed the intensity profile, and clustered proteins were easily distinguished from background fluorescence and random fluctuations in membrane protein surface distribution. Analysis of endogenous Kv2.1 in neurons at 14 DIV and recombinant Kv2.1 in neurons at 9 DIV revealed clear peaks of intense staining that correspond to clusters ranging from 1 to 4 μm in diameter (Figure 1E). Many clusters have double peaks of staining intensity that correspond to a line drawn through a ring-shaped cluster. Intercluster signals are minimal relative to those found in peaks, emphasizing efficient targeting of both endogenous and recombinant Kv2.1 to these membrane clusters. In contrast, intensity profiles of Kv2.2 staining in Kv2.2-transfected neurons had randomly fluctuating patterns, with very little evidence of clustering (Figure 1E). Intensity profiles of MAP2 staining showed relatively homogenous staining patterns.

Truncation Mutants Reveal Parallels between Kv2.1 Targeting in Neurons and Epithelial Cells

We next examined the subcellular localization and clustering of two epitope-tagged C-terminal truncation mutants, HA-ΔC187, lacking the last 187 amino acids of the 853–amino acid Kv2.1 polypeptide (i.e., containing a.a. 1–666), and HA-ΔC318, lacking the last 318 amino acids (i.e., containing only a.a. 1–535). Previous studies in MDCK cells showed that ΔC187 was localized similar to wild-type Kv2.1 (in clusters in lateral membranes), while ΔC318 was expressed uniformly throughout apical and lateral membranes (Scannevin et al., 1996).

In HA-ΔC187-transfected neurons, 12CA5 staining was observed in large clusters on cell bodies and proximal dendrites (Figures 2A and 2C). This subcellular distribution was similar to that of recombinant and endogenous wild-type Kv2.1, suggesting that in hippocampal neurons, as well as in MDCK cells, amino acids 666–853 of Kv2.1 do not contain a PRC signal. We also cotransfected neurons with wild-type Kv2.1 and HA-ΔC187 and double stained with 12CA5 and polyclonal "KC" antibody (Trimmer, 1991). HA-ΔC187 and HA-ΔC318 are not recognized by KC antibody, as they lack the KC epitope (Kv2.1 a.a. 837–853). Double staining of HA-ΔC187/Kv2.1-cotransfected neurons shows precise colocalization of HA-ΔC187 (12CA5, red) and wild-type Kv2.1 (KC, green) staining, shown by yellow staining in large clusters on the soma and proximal dendrites, with very few separate red or green signals (Figure 2C). These data suggest that wild-type Kv2.1 and HA-ΔC187 can coexist in the same clusters and further emphasize that overexpression of these two constructs did not overwhelm the PRC machinery of these hippocampal neurons.

HA-ΔC318 had a strikingly different localization in that HA-ΔC318 immunostaining (green) was uniformly distributed over the entire neuron (Figure 2B). Staining was observed throughout the extent of axons (indicated by an arrow) and dendrites, with no obvious clustering (Figure 2B). Some HA-ΔC318 staining (indicated by an arrowhead) was found even more distally on dendrites than staining (red) for MAP2 (Figure 2B). A subset of HA-ΔC318-transfected neurons also exhibited faint but visible ring-shaped structures on cell bodies that are similar in appearance to endogenous Kv2.1 clusters.

In order to investigate whether this low level of HA-ΔC318 clustering was due to heteromeric assembly between introduced HA-ΔC318 and endogenous Kv2.1, we overexpressed both HA-ΔC318 and wild-type Kv2.1 in the same neurons. We now found much more extensive overlap (yellow) of HA-ΔC318 staining (red) with

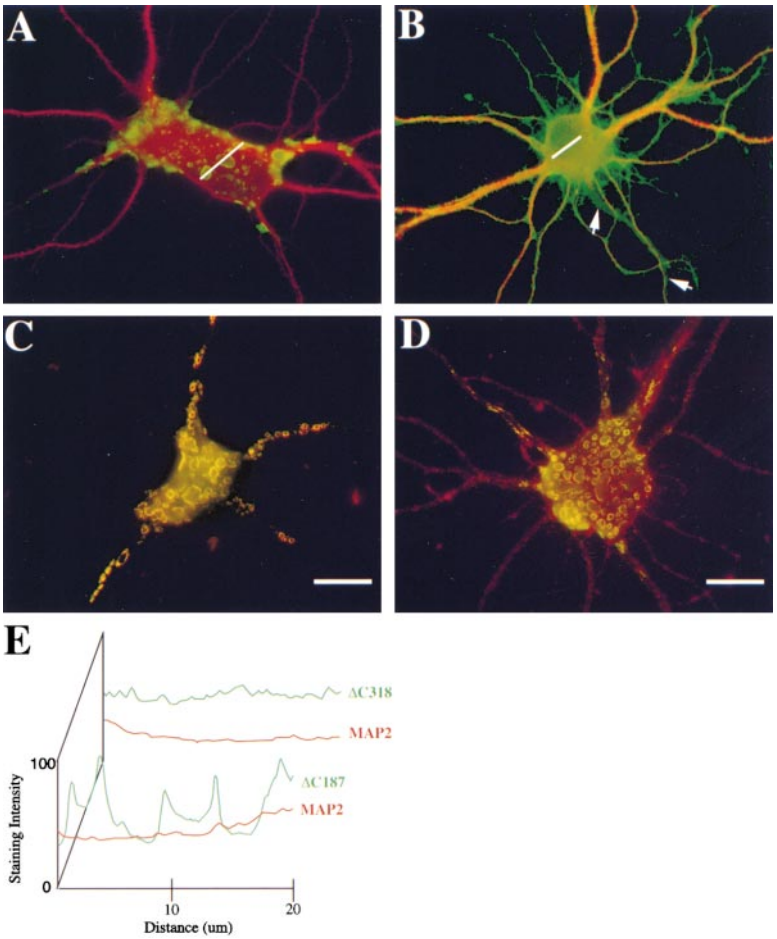

Figure 2. Truncation Mutants Localize the Kv2.1 PRC Signal to the C Terminus

(A) The distribution of HA-ΔC187 in neurons. Staining for HA-ΔC187 (green) was restricted to clusters on the soma and proximal dendrites that were identified by MAP2 labeling (red). HA-ΔC187 was also clustered similar to wild-type Kv2.1.

(B) The nonpolarized uniform distribution of HA-ΔC318. In contrast to HA-ΔC187, the HA-ΔC318 truncation mutant did not exhibit a polarized or a clustered distribution. In addition to somatodendritic staining, HA-ΔC318 staining (green) was present throughout the axon (indicated by an arrow) that was identified as negative for MAP2 staining (red). Note that the tips of dendrites and dendritic filopodia also appear green due to HA-ΔC318 staining in these areas of relatively low concentrations of MAP2 (indicated by arrowheads).

(C) Cotransfection of wild-type Kv2.1 and HA-ΔC187. Cells were transfected with wild-type Kv2.1 and HA-ΔC187 and immunostained for Kv2.1 (green) and HA-ΔC187 (red). Wild-type Kv2.1 and HA-ΔC187 colocalized and coclustered are shown as the yellow color due to the overlap of the green and red fluorescence signals.

(D) Cotransfection of wild-type Kv2.1 and HA-ΔC318. In contrast to (C), cotransfection of wild type (green) and HA-ΔC318 (red) revealed overlap of HA-ΔC318 (red) and clustered Kv2.1 signals on the soma (yielding yellow clusters) and nonoverlap on more distal portions of the dendrites, which exhibit only HA-ΔC318 (red) staining. Scale bar, 20 μm.

(E) Plots of relative staining intensity. The intensity of cell body staining was measured over a 20 μm segment of neurons, indicated by the white bars in (A) and (B).

clustered wild-type Kv2.1 (green) staining, although some nonoverlapping uniform HA-ΔC318 (red) staining was still observed throughout the neuron. We next performed experiments in which we altered ratios of the two cDNAs in transfection cocktails and found that increasing HA-ΔC318 cDNA yielded more uniform HA-ΔC318 staining, whereas increasing wild-type Kv2.1 cDNA caused HA-ΔC318 staining to become clustered (data not shown). Interestingly, staining of neither endogenous nor overexpressed wild-type Kv2.1 was altered by HA-ΔC318 overexpression, showing that this mutant is not a dominant-negative for targeting and does not have any alternative clustering information that interferes with the Kv2.1 PRC and that the PRC signal on Kv2.1 is sufficient to confer clustering to heteromultimeric channels. Thus, while analysis of targeting mutants in these cultured hippocampal neurons is somewhat complicated by the presence of low levels of endogenous Kv2.1, we can use overexpression to drive formation of homomeric mutant channels that exhibit their inherent targeting phenotype. It should be noted that as Kv2.1 is expressed in virtually all neurons (Rhodes et al., 1997), we cannot do experiments in neurons in the absence of endogenous Kv2.1.

Analysis of staining intensity profiles of HA-ΔC187 (Figure 2E) shows similar patterns to that seen in Figure 1D for endogenous and recombinant Kv2.1. There are clear paired peaks of intense staining that correspond to the donut-shaped clusters, with comparatively low levels of staining in intercluster regions (Figure 2E). In contrast, HA-ΔC318 shows a relatively homogenous staining pattern, with little suggestion of clustering observed for Kv2.1 (Figure 2E). These results together show that, as in MDCK cells (Scannevin et al., 1996), amino acids 536–666 are required for targeting and clustering of Kv2.1.

The C-Terminal Cytoplasmic Domain of Kv2.1 Acts as a Signal for Restricted Proximal Localization and Clustering of Kv2.2

Rat Kv2.1 and Kv2.2 exhibit 66% overall amino acid identity, including 87% identity throughout the N terminus, the core transmembrane region, and the initial portion of the C terminus. At Kv2.1 amino acid 521, there is a profound divergence, after which the two proteins share only 24% identity (Figure 3A). By comparison, this region of Kv2.1 is highly conserved between rat and human Kv2.1 (86.7% amino acid identity), even though deletion of this entire region yields functional channels (VanDongen et al., 1990; Scannevin et al., 1996; Murakoshi et al., 1997). Similarly, this C-terminal region (a.a. 520–803) of rat Kv2.2 is highly conserved among Kv2.2

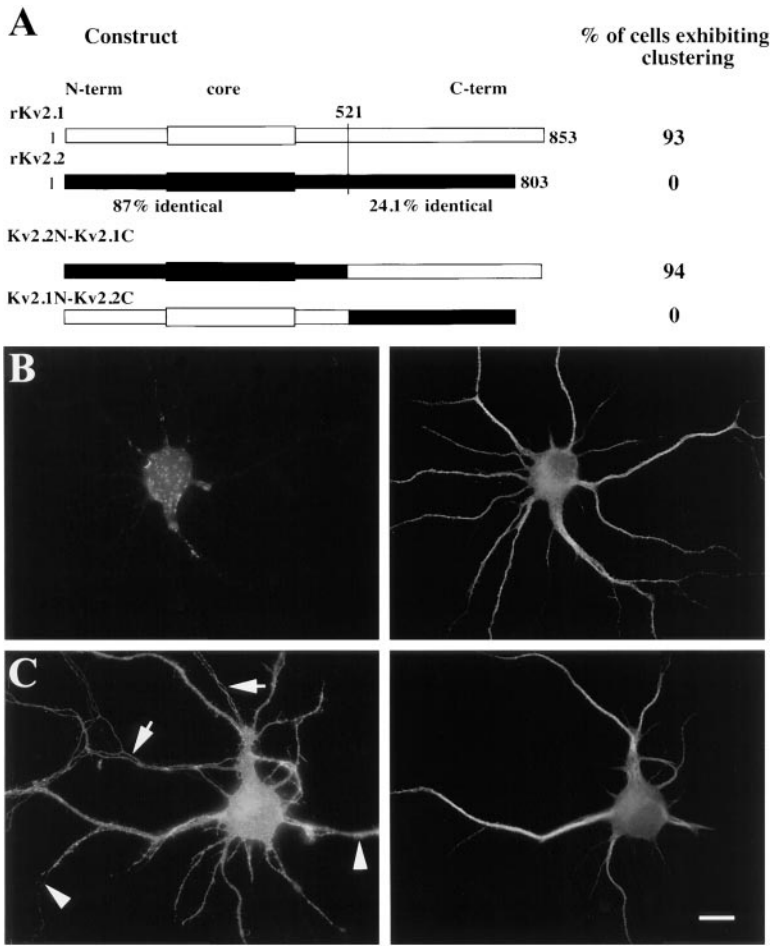

Figure 3. Chimeras between Kv2.1 and Kv2.2 Show Sufficiency of the Kv2.1 C Terminus to Serve as a PRC Signal

(A) Schematic diagrams of Kv2.1, Kv2.2, and the two chimeras. Kv2.1 sequences are shown in black and Kv2.2 sequences in white. Percentage of cells exhibiting clustering was determined by collecting images from 50 transfected cells from each of two independent experiments; values presented represent the average from these two independent experiments.

(B) Staining for the Kv2.2N-Kv2.1C chimera shows a distribution to cell bodies and proximal dendrites in clusters, similar to that of wild-type Kv2.1. The right panel shows MAP2 staining of the cell in the left panel.

(C) Staining for the Kv2.1N-Kv2.2C chimera reveals a nonpolarized and uniform distribution. Axonal staining is indicated by arrows. Note that the extent of staining for Kv2.1N-Kv2.2C at the tips of dendrites and dendritic filopodia (indicated by arrowheads) is greater than for the MAP2 staining in the right panel. Scale bar, 20 μm.

orthologs. Having determined through analyses of HA-ΔC318 and HA-ΔC187 (presented above) that a portion of the cytoplasmic C terminus of Kv2.1 was required for an active PRC signal, we next addressed whether the Kv2.1 C-terminal region could act as a PRC signal for Kv2.2.

Transfer-of-function experiments were carried out by generating C-terminal chimeras between Kv2.1 and Kv2.2. The C-terminal 332 amino acids of Kv2.1 (a.a. 522–853) were used to replace the analogous region (a.a. 520–803) of Kv2.2 to generate the Kv2.2N-Kv2.1C chimera; we also made the reciprocal chimera, Kv2.1N-Kv2.2C (Figure 3A). These chimeric proteins were expressed in hippocampal neurons and assayed for localization. The Kv2.2N-Kv2.1C chimera exhibited restricted proximal dendritic localization and clustering like wild-type Kv2.1, not diffuse somatodendritic expression like Kv2.2 (Figure 3B). The K2.2N-Kv2.1C chimera colocalized with both endogenous Kv2.1 and coexpressed recombinant wild-type Kv2.1 (data not shown). Thus, the C terminus of Kv2.1 acts as a Kv channel PRC signal in that it is sufficient to redirect targeting of Kv2.2. The Kv2.1N-Kv2.2C chimera had a nonclustered localization and was present throughout the extent of the dendritic arbor (Figure 3C) in a pattern similar to that observed for wild-type Kv2.2 (Figure 1C). These data together show that amino acids 522–853 of Kv2.1 are not only necessary but also sufficient to act as a PRC signal for Kv2 channels in hippocampal neurons and that contrasting subcellular localizations of these two neuronal K^+ channels observed in brain (Hwang et al., 1993) are determined by corresponding C-terminal domains.

Identification of a Minimal Targeting and Clustering Domain on Kv2.1

To further dissect the 131–amino acid domain that differentiates HA-ΔC187 and HA-ΔC318 and contains the active PRC signal, a series of internal deletion and C-terminal truncation mutants of Kv2.1 were expressed in hippocampal neurons. First, an internal deletion mutant, HA-Δ536-665, that eliminates the domain that distinguishes HA-ΔC187 from HA-ΔC318 was generated and expressed. HA-Δ536-665 was localized throughout the entire cell body, dendrites, and axon, in a pattern identical to HA-ΔC318. While a low level of HA-Δ536-665 clustering on cell bodies was observed, this was presumably due to heteromultimerization with endogenous Kv2.1, and staining was relatively high and uniform between clusters (Figure 4A). This finding indicates the existence of the Kv2.1 PRC signal between amino acids 536–665.

To further localize the domain necessary for proximal restriction and clustering, we expressed HA-tagged Δ521-573 and Δ609-665 internal deletion mutants, which

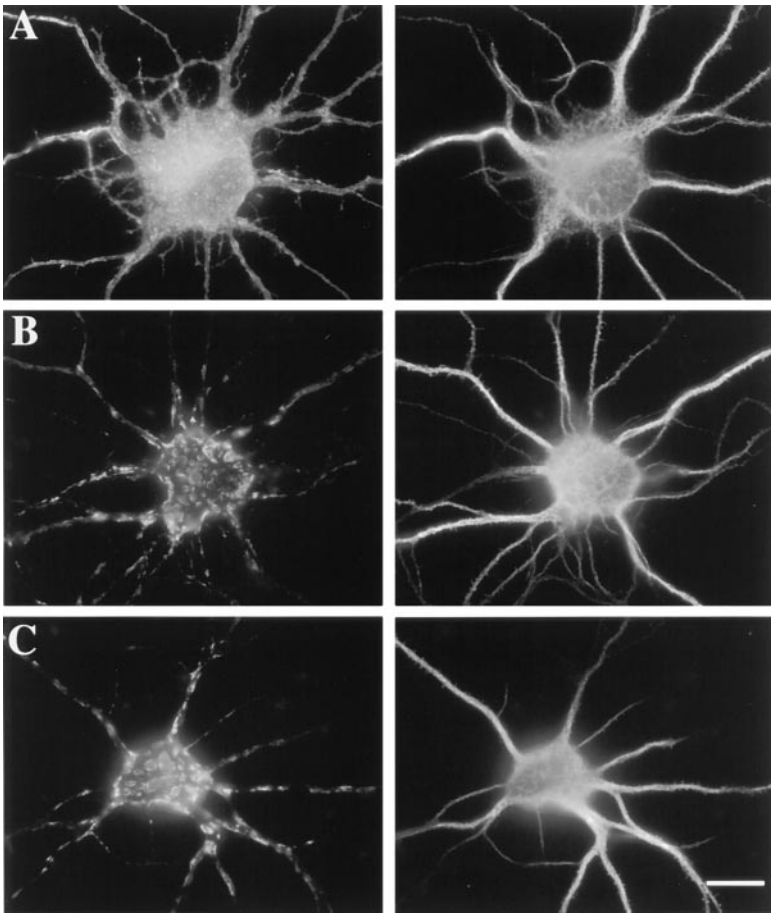

Figure 4. Analysis of Internal Deletion Mutants

(A) Staining for the Δ536-665 internal deletion mutant reveals a generally uniform localization extending throughout both the dendritic and axonal arbor.

(B and C) Staining for the Δ521-573 and Δ609-665 internal deletion mutants resembles that of clustered wild-type Kv2.1, although the mutant clusters extend farther out on the dendrites. The panels on the right show MAP2 staining of the cells in the left panels.

Scale bar, 20 μm.

exhibited clustered localization on the soma and dendrites similar to HA-Kv2.1 and HA-ΔC187 (Figures 4B and 4C), although some additional Δ609-665 clusters were also seen more distally on the dendrites. From these internal deletion mutants, we concluded that the PRC signal is located in a 37–amino acid segment (a.a. 573–609).

To further define the minimal region of Kv2.1 that can act as a PRC signal in neurons, we expressed a panel of progressive C-terminal truncation mutants, using a PCR mutagenesis strategy to generate five distinct truncation mutants between amino acids 580 and 616. Neurons were transiently transfected with these HA-tagged C-terminal truncation mutants ΔC237 (a.a. 1–615), ΔC247 (a.a. 1–605), ΔC254 (a.a. 1–598), ΔC263 (a.a. 1–589), and ΔC273 (a.a. 1–579). Staining of ΔC237, ΔC247, and ΔC254 with 12CA5 antibody revealed the presence of a PRC signal in that staining was identical to that of wild-type Kv2.1. An example of such staining, in this case for ΔC254, is shown in Figure 5A. Two constructs that had larger regions deleted, ΔC263 and ΔC273, displayed nonpolarized and homogenous localization similar to HA-ΔC318, as shown in Figure 5B for ΔC263. Staining extended throughout the dendritic arbor, even slightly beyond MAP2 staining, and axonal staining was evident to the distal axon, and no clustering was obvious. These data taken together with analyses of internal deletion mutants localize the Kv2.1 PRC signal to a 26–amino acid segment (a.a. 573–598) of the 853–amino acid Kv2.1 polypeptide (Figure 5C).

Point Mutants Identify Critical Amino Acids within the Minimal Targeting Domain

To further define residues within this minimal domain, we used site-directed alanine scanning mutagenesis to mutate each amino acid from 573 to 598 to alanine, with the exception of A597 (to threonine; Figure 6A). Residues I576 and I591 were each mutated to both alanine and threonine, and residue E596 to alanine and lysine. Each of these mutant polypeptides was expressed in hippocampal neurons and assayed for subcellular distributions, which fell into two groups, those that had a restricted proximal and clustered localization similar to Kv2.1 and those that had a nonpolarized and uniform distribution similar to ΔC318. Mutation of residues S583, S586, F587, and S589 to alanine was found to exhibit a nonpolarized and uniform distribution similar to HA-ΔC318; an example of such staining (for S583A) is shown in Figure 6B. The remainder of the point mutants exhibited polarized and clustered subcellular distributions identical to Kv2.1, as shown in Figure 6C for mutant protein S582A. These results show that within the 26–amino acid minimal targeting domain, four positions (S583, S586, F587, and S589) are absolutely necessary for the polarized and clustered distribution of Kv2.1 observed in mammalian central neurons.

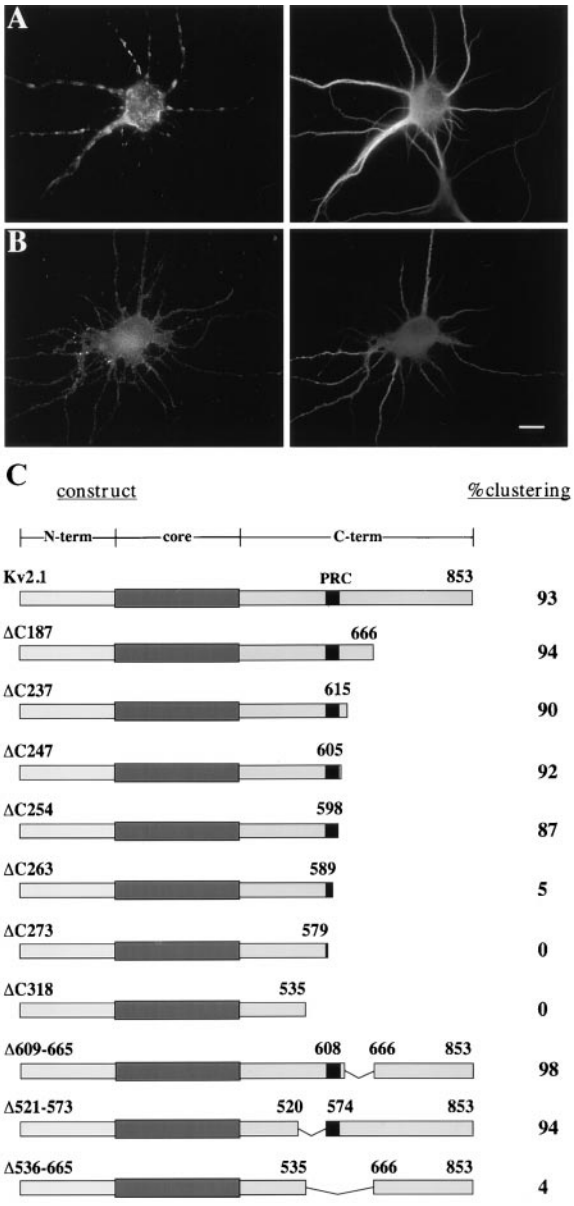

Figure 5. A Series of C-Terminal Truncated Mutant Proteins Demonstrates that the PRC Signal Is Localized to a 26–Amino Acid Sequence

(A) A truncation mutant protein, ΔC254, lacking the last 254 amino acids of Kv2.1 was present in cell bodies and dendrites and was clustered.

(B) The ΔC263 truncation mutant, which lacks the last 263 amino acids of Kv2.1, is expressed in a uniform and nonpolarized manner. Right panels show MAP2 staining of the cells in left panels. Scale bar, 20 μm.

(C) Schematic diagram of the Kv2.1 C-terminal truncation and internal deletion mutants used to define the minimal PRC signal. Percentage of cells exhibiting clustering was determined by collecting images from 50 transfected cells from each of two independent experiments; values presented represent the average from these two independent experiments.

A 65–Amino Acid Fragment of Kv2.1 Containing the Minimal PRC Domain Is Able to Cluster the Kv1.5 K^+ Channel but Not Type I Membrane Proteins

We next addressed whether the C-terminal domain of Kv2.1 that contains the identified PRC signal could confer targeting on heterologous proteins. We initially appended the entire Kv2.1 C terminus to type I membrane proteins that have been used previously as backbones for targeting studies, namely CD44 (hyaluronan receptor; Neame and Isacke, 1993) and Tac (interleukin 2 receptor α chain; Humphrey et al., 1993). Neither of these model type I membrane protein backbones could be redirected to, or clustered on, the soma and proximal dendrites by appending the Kv2.1 cytoplasmic tail to their C termini (data not shown). These data suggest that the PRC signal contained in the cytoplasmic tail of Kv2.1 may have specific structural requirements provided by the Kv2.2 backbone but not by the backbone of these type I membrane proteins.

One structural feature that is characteristic of Kv channels, and not shared by these other model protein backbones, is assembly into tetramers (MacKinnon, 1991). To test the role of tetramerization in the function of the PRC signal in the Kv2.1 C terminus, we generated a chimeric protein using the *Shaker*-related or Kv1 family member Kv1.5. Kv1.5 shares only 27% overall amino acid identity with Kv2.1, similar to the extent of CD44 (26%) and Tac (18%) identity with Kv2.1. We first generated a truncation mutant of Kv1.5, Kv1.5Δ10, in which the last ten amino acids, including the consensus PDZ-binding motif at the very C terminus, were deleted (i.e., this mutant contained a.a. 1–593 of Kv1.5). This was to ensure that no Kv1.5 clustering due to interaction with endogenous PDZ domain–containing proteins such as PSD-95 would occur. Kv1.5Δ10 expressed in transfected neurons was found throughout the cell, including the extent of the dendritic and axonal processes, and was not clustered (Figure 7A). When amino acids 536–600 of Kv2.1, including the minimal PRC domain (a.a. 573–598), were appended to the C terminus of Kv1.5Δ10, the chimeric protein (a.a. 1–593 of Kv1.5 and 536–600 of Kv2.1) was now targeted to the soma and proximal dendrites in donut-shaped clusters (Figure 7B). Unlike wild-type Kv2.1, this chimeric protein was also clustered in more distal portions of axons (arrows in Figure 7B), suggesting that information for axonal targeting, presumably derived from Kv1.5, was present in this chimera. These results demonstrated that of the polypeptide backbones examined, only Kv channels were capable of responding to the PRC signal present in the C terminus of Kv2.1, perhaps due to a functional requirement for tetramerization of this signal.

Discussion

A Novel Dendritic Targeting and Clustering Signal

The *cis*-acting PRC signal discussed here is a distinct targeting or clustering signal for neuronal proteins. The PRC signal sequence has a net negative charge of −4 (five acidic residues and one basic residue) and is rich in serine and threonine residues (7/26 positions). Three of four critical positions identified in the alanine scan are serine residues; loss of function seen upon the simple

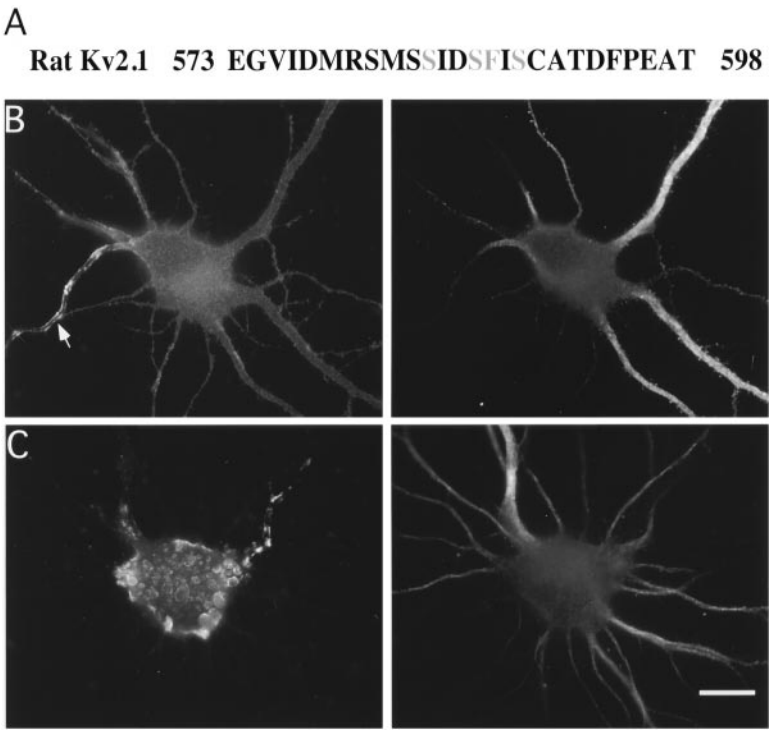

Figure 6. A Mutational Analysis within the Minimal PRC Signal Identifies Four Key Amino Acid Residues

(A) Sequence of the minimal domain for the Kv2.1 PRC signal as defined by internal deletion and truncation mutants. Mutations in positions shown in black yield no change in localization, while those in gray resulted in the loss of both polarized expression and clustering.

(B and C) One representative of each of these classes of mutants is shown.

(B) S583A (essential to PRC signal). Axon is indicated by an arrow.

(C) S582A (nonessential to PRC signal). The right panels in (B) and (C) show MAP2 staining of the cells in the left panels.

Scale bar, 20 μm.

removal of the hydroxyl group by serine to alanine mutations suggests a critical role for the hydroxyl groups themselves, perhaps through participation in hydrogen bonds. These residues could play critical roles in intramolecular hydrogen bonding important in stabilizing this domain of Kv2.1 or in intermolecular hydrogen bonds mediating protein–protein interactions involved in Kv2.1 localization.

Interaction with PDZ domain–containing proteins is one means of targeting and clustering of ion channels in neurons (Sheng and Pak, 1999). For instance, the membrane-associated guanylate kinase, Discs-large, is necessary for correct localization of *Shaker* Kv channels and fasciclin at *Drosophila* neuromuscular junctions (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997). Class I PDZ domains, such as those present in PSD-95, recognize the peptide motif containing E-S/T-X-V/I (where X is any amino acid) at the C terminus (Kim et al., 1995), and class II PDZ domains, such as those of CASK, recognize a peptide motif with hydrophobic or aromatic side chains as the three C-terminal residues (Songyang et al., 1997). As the Kv2.1 PRC signal functions internally and as there are no identifiable class I or class II PDZ-interacting motifs within the PRC signal

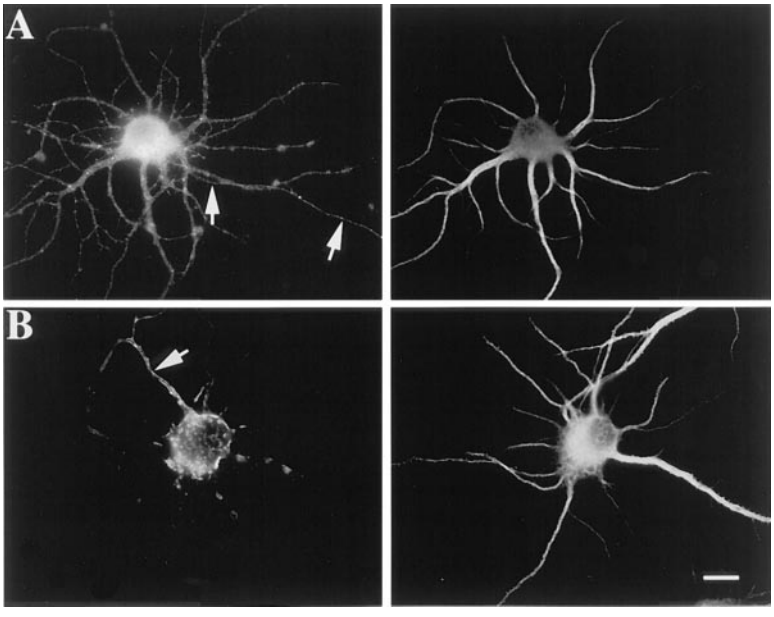

Figure 7. A Kv1.5–Kv2.1 Chimera Containing a 65–Amino Acid Kv2.1 Fragment Including the Minimal PRC signal Is Clustered

(A) A C-terminal truncation mutant of Kv1.5, Kv1.5Δ10, lacking the last ten amino acids, and the Kv1.5 PDZ-binding motif was expressed throughout transfected neurons.

(B) Appending 65 amino acids (536–600) of Kv2.1 onto the C terminus of Kv1.5Δ10 results in somal and proximal dendritic restriction and clustering. Note additional clustered axonal staining was not observed for Kv2.1 (arrows). The right panels in (A) and (B) show MAP2 staining of the cells in the left panels.

Scale bar, 20 μm.

(or in the nonessential C terminus of Kv2.1), we can assume that the PRC signal directs targeting and clustering of Kv2.1 by a mechanism other than interaction with these conventional PDZ domains. In addition, localization of Kv2.1 in mammalian neurons is distinct from that described for neuronal channel-clustering proteins PSD-95/SAP90 (Laube et al., 1996), SAP97 (Muller et al., 1995), chapsyn-110/PSD-93 (Brenman et al., 1998), and SAP102 (Muller et al., 1996). However, since other motifs that interact with PDZ domains will surely be found (Hata et al., 1998), we can not rule out the possibility that the Kv2.1 PRC signal acts through an interaction with a PDZ domain–containing protein.

The PRC sequence identified here is also distinct from known targeting signals for polarized epithelial proteins (Keller and Simons, 1997). This PRC signal does not contain a tyrosine or dileucine typical of endosomal or basolateral sorting motifs that direct targeting to dendritic endosomes and plasma membranes (de Hoop et al., 1995; West et al., 1997; Jareb and Banker, 1998). Examination of the minimal 26–amino acid sequence has revealed no similarity to common or conserved basolateral or somatodendritic localization sequences (Dargemont et al., 1993; Jareb and Banker, 1998; Stowell and Craig, 1999). This is consistent with a number of previous studies in epithelial cells and neurons that have led to a consensus that basolateral/somatodendritic targeting signals are based more on conformation than primary sequence (discussed in Trimmer, 1999). It should be stressed that the minimal PRC signal sequence is not present in any other proteins in the database, including those that have been reported to exhibit a Kv2.1-like localization (e.g., ryanodine receptors, dihydropyridine-sensitive Ca^{2+} channels; see below).

One other Kv2 or *Shab*-related Kv channel subfamily member, Kv2.2, has been identified in mammals. Kv2.2, however, has a distinct, nonoverlapping distribution from that of Kv2.1 in the same brain neurons (Hwang et al., 1993; Maletic-Savatic et al., 1995), and thus presumably does not form heteromultimeric channels in these cells, as do members of the Kv1 or *Shaker*-related subfamily (Rhodes et al., 1997; Shamotienko et al., 1997). The differences in subcellular distributions of Kv2.1 and Kv2.2 in the same neurons are now explained by our analysis of Kv2.1-Kv2.2 chimeras, which clearly demonstrates that divergent cytoplasmic C termini of these two highly related proteins determine their contrasting subcellular distributions.

Based on our analyses of localization of Kv2.1, Kv2.2, and Kv2.1-Kv2.2 chimeras, we would predict that the Kv2.1 PRC sequence itself would not be well conserved in Kv2.2. Interestingly, we find that this domain is quite similar in Kv2.2, with 17 out of 26 amino acids in the Kv2.1 PRC sequence conserved in Kv2.2, including the four key residues defined in our alanine scan. Again, this is consistent with the model that somatodendritic targeting signals may be based more on overall conformational considerations than on primary sequence (Trimmer, 1999). In fact, the predicted secondary structure of the 26–amino acid Kv2.1 PRC signal includes a β sheet for the segment (EGVIDMRS) just preceding the four key residues identified in the alanine scan. This β sheet is not predicted for the analogous segment (EVIVDMKS) of Kv2.2. In addition, that a 60–amino acid fragment containing the Kv2.1 PRC signal can operate in a much less related Kv channel, Kv1.5, suggests that information needed for autonomous PRC function is contained within this fragment. However, the C terminus of Kv2.1 could not direct targeting of two type I membrane proteins tested, suggesting that tetramerization may be necessary for PRC function. It remains a goal to determine the structural context in which the Kv2.1 PRC signal mediates Kv2.1-specific intra- or intermolecular interactions critical in determining its precise targeting in neurons. Detailed structural information may be needed to resolve this interesting enigma.

An additional factor that may contribute to differential distribution of Kv2.1 and Kv2.2 is phosphorylation. Phosphoamino acid analysis of Kv2.1 labeled with ^{32}P in vivo indicated that all detectable labeling was on serine and that the bulk of ^{32}P labeling was absent in the mislocalizing ΔC318 mutant (Murakoshi et al., 1997). It is interesting that 5 of 26 amino acids in the PRC sequence are serines, including three of four key positions identified in the alanine scan. Although these key serine residues are not in consensus phosphorylation sites for known kinases, it raises the intriguing possibility that subcellular localization of Kv2.1, like its voltage-dependent activation (Murakoshi et al., 1997), can be regulated by phosphorylation through the covalent modification of the Kv2.1 PRC signal and that phosphorylation can dynamically regulate aspects of both Kv2.1 function and localization.

Potential Mechanisms and Functional Implications of Kv2.1 Localization

The PRC signal on Kv2.1 must act during polarized targeting to dendrites, restriction to very proximal portions of these dendrites, and sequestration into high-density clusters. The precise mechanism whereby the PRC signal on Kv2.1 functions in these events is as yet unknown. However, data presented here and in our previous studies allow us to propose that heterophilic interaction of the Kv2.1 PRC with as yet unknown intracellular proteins, as opposed to homophilic interaction of multiple Kv2.1 tetramers (e.g., during clustering), underlies these events. Our studies predict that there exist in neurons intracellular proteins that can specifically interact with the Kv2.1 PRC signal and direct Kv2.1 localization. These proteins, or functional homologs, are present in each diverse cell type that exhibits clustering of either endogenous or introduced Kv2.1, i.e., mammalian central neurons, PC12 cells (Sharma et al., 1993), and MDCK cells (Scannevin et al., 1996). It also follows that at least some components must be lacking in cell types which do not cluster Kv2.1, including astrocytes and COS-1 (Shi et al., 1994; Scannevin et al., 1996), HEK 293, and L(tk-) tissue culture cells.

Association with such PRC-specific interacting proteins would cause retention of Kv2.1 at specific sites, resulting in clustering and restriction to the soma and proximal dendrites. Many variations on a theme of general targeting and specific clustering/retention are possible, for example, those involving a more directed targeting and insertion of Kv2.1 in the neighborhood of final clustering sites on the soma and proximal dendrites. Future studies combining expression of fluorescent Kv2.1 isoforms in cultured neurons with real-time

imaging of targeting and clustering will allow for more detailed insights into the contribution of directed targeting and focal retention to the precise localization of Kv2.1.

It should be noted that Kv2.1 clusters observed in hippocampal neurons are distinct in morphology (larger and donut- or ring-shaped) from those observed in MDCK cells (smaller and solid in appearance; Scannevin et al., 1996). This may indicate that neuronal-specific components exist that are necessary for creating the precise morphology of neuronal Kv2.1 clusters. Moreover, it is clear from these studies that the clustering machinery in neurons has a capacity that greatly exceeds normal expression levels of Kv2.1, as overexpression of Kv2.1 with viral promoters leads to efficient Kv2.1 clustering. Our identification of the Kv2.1 PRC domain provides a powerful tool for future studies aimed at identifying the individual components of what is clearly a novel neuronal ion channel clustering mechanism.

Immunogold electron microscopy studies have shown that clusters of Kv2.1 in rat brain cortical neurons are associated with two distinct types of plasma membrane specializations (Du et al., 1998). Plasma membrane Kv2.1 clusters are found at somatodendritic contact points for astrocytic processes, where specialized cell–cell interactions between apposed neuronal and glial membranes may mediate a number of functions. It has been suggested that clustering of Kv2.1 at these sites may allow for more efficient astrocyte-mediated buffering of K^+ ion efflux through Kv2.1 channels (Du et al., 1998). It is clear from the studies presented here on hippocampal neurons cultured in the absence of physical contact with astrocytes that neuron–astrocyte contact in itself is not a primary determinant of Kv2.1 clustering. However, Kv2.1 clustering in cultured hippocampal neurons may be related to another class of contact zones, those on basal neuronal membranes where cell–substratum contact occurs in culture. Interestingly, previous studies in PC12 (Sharma et al., 1993) and MDCK (Scannevin et al., 1996) cells showed that cell–cell contact was required for Kv2.1 clustering. This may indicate a generality in the requirement of an extracellular influence in the form of cell–cell or cell–substratum contact for Kv2.1 clustering, perhaps suggesting a complex macromolecular organization for these domains analogous to the cell–cell contacts at the synapse (Kim and Huganir, 1999).

A second membrane specialization observed at sites of Kv2.1 clustering in rat brain cortical neurons was subsurface cisternae (Du et al., 1998). These have been proposed to be specialized intracellular endoplasmic reticulum–derived organelles important in non-IP_3- (i.e., ryanodine receptor) mediated Ca^{2+} release (Garaschuk et al., 1997). In cultured hippocampal neurons, these organelles are localized to the soma and proximal dendrites (Korkotian and Segal, 1997). Interestingly, dihydropyridine-sensitive Ca^{2+} channels have also been reported to be clustered on the soma and proximal dendrites of mammalian central neurons (Hell et al., 1993), suggesting that Kv2.1 channels and Ca^{2+} signaling through both plasma membrane entry and release from intracellular stores may be spatially associated. It is interesting to speculate that clustering of Kv2.1 at or near these sites reflects a role for Kv2.1 in regulation of neuronal Ca^{2+} signaling and/or in regulation of Kv2.1 activity, through, for example, Ca^{2+}-mediated phosphorylation. Kv2.1 exhibits high-level and widespread expression in mammalian brain, and its role has been established as a major component of delayed rectifier K^+ current in rat hippocampal neurons (Murakoshi and Trimmer, 1999) and of slowly deactivating current critical to discharge patterning in rat globus pallidus neurons (Baranauskas et al., 1999). The PRC signal identified here targets Kv2.1 such that it is poised to play a pivotal and dynamic role in regulating transmission of electrical signals into and out of proximal dendrites and soma. Given recent appreciation of the importance of somal and dendritic Kv channels in regulating both synaptic currents and backpropagating action potentials (Stuart et al., 1997) and the impact that differential spatial distribution of dendritic Kv channels can have on dendritic excitability (Hoffman et al., 1997), it is clear that precise localization of Kv2.1 on the soma and proximal dendrites may be a key to its function. The function of Kv2.1 in neurons is presumably distinct from that of Kv2.2, not due to differences in their inherent biophysical characteristics (which are very similar) but due to differential targeting and localization conferred by the Kv2.1 PRC localization signal.

Experimental Procedures

Primary Hippocampal Cultures

Hippocampal cultures were prepared as previously described (Banker and Cowan, 1977; Murakoshi and Trimmer, 1999) with the following modifications. Coverslips were placed in wells face up but did not contact the glia due to the presence of paraffin wax pedestals. Most (>85%) of the neurons in these cultures developed the characteristic morphology of hippocampal pyramidal cells.

Generation of Kv2.1 and Mutant Kv2.1 Expression Plasmids

Generation of the pKv2.1/RBG4 expression plasmid (Shi et al., 1994) and the Kv2.1 expression plasmid with the N-terminal HA tag, pKv2.1/CGN (Sharma et al., 1993), was previously described. Construction of pHA-ΔC187/RBG4 and pHA-ΔC318/CGN was also previously described (Scannevin et al., 1996).

To generate the internal delation mutant Δ536-665, Kv2.1/RBG4 was digested with TthIII1 at nucleotide 1607 and with Eco47III at nucleotide 1997. The 5′ overhangs were treated with the Klenow fragment of DNA polymerase I to generate blunt ends and with calf intestinal phosphatase to prevent religation. This fragment was ligated with SmaI linkers (New England Biolabs) and then digested with SmaI and religated to generate the necessary frame shift between blunted TthIII1 and Eco47III.

To generate Δ609-665, a PCR fragment was amplified from the Kv2.1 ApaI site to a blunt-ended primer ending at nucleotide 1824. Δ609-665 was constructed by ligating this PCR fragment into ApaI- and Eco47III-digested pKv2.1/CGN. To generate Δ521-573, two PCR fragments were generated. The first PCR fragment was amplified from nucleotide 961 to 1560. An EcoRI site was inserted into the downstream cloning site, and this fragment was digested with NruI and EcoRI. The second PCR fragment was amplified from nucleotide 1720 to 2040. An EcoRI site was inserted at base pair 1720. This construct was digested with EcoRI and Eco47III. These PCR fragments were ligated into Kv2.1/CGN that had been digested with NruI and Eco47III.

A series of progressive C-terminal truncation mutants was constructed employing a PCR-based mutagenesis approach. This technique utilized a unique ApaI site in the Kv2.1 cDNA at nucleotide 770 as the upstream cloning site and a unique AflII site at Kv2.1 nucleotide 2409 as the downstream cloning site. A series of 3′ PCR primers were synthesized that hybridized to the sequence in the Kv2.1 C-terminal region up to the base pair where the deletion

mutant was to end. After this region of homology, a TGA stop codon was introduced into the primer sequence and followed by either a blunt end (Δ237, Δ263, Δ273) or an AflII restriction site (Δ247, Δ254). Using a 5′ primer just upstream of the ApaI site and pKv2.1/RBG4 as a template, a fragment was amplified by PCR using the mutant 3′ primers. The resultant fragments contained the upstream ApaI site, an in-frame truncated Kv2.1 coding region, and a stop codon directly after the last Kv2.1 codon in the mutant followed by either a blunt end or an AflII site. The Kv2.1 cDNA and the truncated PCR product were digested with ApaI and AflII. Each of these was ligated to the 5461 base pair Kv2.1 fragment ApaI to AflII (or blunt). Point mutations were introduced using the Quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA). DNA sequence fidelity of all constructs and PCR inserts was verified by sequencing.

Generation of Kv2.1N-Kv2.2C and Kv2.2N-Kv2.1C Chimeras

To generate Kv2.1N-Kv2.2C, two separate PCR reactions were used to generate the final chimeric construct. The first reaction amplified Kv2.1 from nucleotide 1 to 1563, and the amplified fragment contained a unique SphI site. This PCR fragment was digested with ClaI and SphI. To generate the Kv2.2C fragment, the reaction amplified Kv2.2 from nucleotide 1157 to 2409. The 5′ and 3′ primers were synthesized such that the amplified PCR fragment contained a unique SphI site and AccI site, respectively. The PCR fragment was then digested with SphI and AccI and inserted into theKv2.1/CGN expression plasmid that had been digested with ClaI and AccI.

To generate Kv2.2N-Kv2.1C-3HA/RBG4, two separate PCR reactions were utilized to generate the final chimeric construct. The first reaction amplified Kv2.2 amino acids 1 to 519. The 5′ and 3′ primers were synthesized such that the amplified PCR fragment contained a unique XbaI restriction site before the start of the coding region and an SmaI restriction site just after amino acid 519. The fragment corresponding to Kv2.1 amino acid 521–853 was amplified by PCR; the 5′ and 3′ primers were synthesized such that the amplified PCR fragment contained the unique SmaI site and ClaI site, respectively. The Kv2.2N PCR fragment was digested with XbaI and SmaI, the Kv2.1C PCR fragment with SmaI and ClaI, and the CGN vector with XbaI and ClaI, followed by a triple ligation. A sequence encoding three tandem copies of HA tag was appended on to the C terminus of Kv2.1.

Generation of Kv1.5Δ10 and the Kv1.5-Kv2.1 Chimera

To generate Kv1.5Δ10, a PCR fragment was amplified from nucleotide 2027 to 2536 of Kv1.5 (Swanson et al., 1990). A synthetic NruI site and a TGA stop codon were introduced into the 3′ primer sequence followed by a downstream BamHI site. This fragment was digested with AccI and BamHI and ligated into Kv1.5/CGN (Nakahira et al., 1996) that was digested with AccI at nucleotide 2083 and BamHI at nucleotide 2569 to generate Kv1.5Δ10.

To generate the Kv1.5-Kv2.1 chimera, a PCR fragment was amplified from Kv2.1 nucleotide 1606 to 1800. Synthetic restriction enzyme sites for NruI and BglII were inserted into the 5′ and 3′ primers, respectively. This PCR fragment was ligated into Kv1.5Δ10 that had been digested with NruI and BamHI to generate the Kv1.5-Kv2.1 chimera.

Neuron Transfection

Cultured hippocampal neurons at 7 days in vitro (DIV) were transfected by the LipofectAMINE Plus method (Life Technologies, Gaithersburg, MD). In brief, 1 μg of DNA was diluted into 100 μl of serum-free transfection medium to which 10 μl of PLUS reagent was added; the solution was mixed and incubated at room temperature for 15 min. Then, 5 μl of LipofectAMINE reagent was diluted into serum-free dilution medium in a second tube and mixed. Precomplexed DNA and diluted LipofectAMINE reagent were combined, mixed, and incubated for 15 min at room temperature. The DNA-PLUS-LipofectAMINE reagent complexes were added to face-up cover slips in individual wells of 6-well tissue culture plates. Complexes were mixed into the medium gently and incubated at 37°C with 5% CO_2 for 48 hr.

In preliminary experiments we expressed GFP and HA-Kv2.1 and examined the time course of expression. Expression of proteins was first detected in a few cells starting at 12 hr posttransfection (the shortest time assayed). The percentage of cells with detectable expression increased markedly between 12 hr and 24 hr with a modest increase between 24 and 48 hr after transfection. The level of protein expression in transfected cells decreased between 48 and 96 hr after transfection. For all remaining experiments, we transfected neurons at 7 DIV and stained at 48 hr posttransfection. At this stage in culture, hippocampal neurons express endogenous Kv2.1, which is polarized and clustered. We used the dendritic marker MAP2 for identifying axonal versus dendritic domains. Axons were identified as MAP2-negative, long, fine-caliber processes that typically extended far beyond the somatodendritic domain.

Immunofluorescence

Immunofluorescence staining of neurons was performed as described (Murakoshi and Trimmer, 1999) with the following antibodies: anti-HA mouse monoclonal IgG_{2b} antibody 12CA5 (BABCO, Berkeley, CA; 1 μg/ml), anti-MAP2 monoclonal IgG_1 antibody (Sigma; 1:4000), anti-Kv2.2 monoclonal IgG_{2a} antibody K37/89 (Epperson et al., 1999; 10 μg/ml), and affinity-purified anti-Kv2.1 rabbit polyclonal antibodies pGEX-drk1 or KC (Trimmer, 1991; 1:50). Immunostaining was visualized by incubation with the following secondary antibodies at 1:2000: fluorescein-5-isothiocynate conjugated, goat anti-mouse IgG_{2b} (Southern Biotechnology Associates, Atlanta, GA) and goat anti-rabbit IgG (Cappell-ICN, Irvine, CA); Texas red conjugated, goat anti-mouse IgG_1 (Southern Biotechnology Associates, Atlanta, GA) and goat anti-mouse IgG (Cappell-ICN, Irvine, CA).

The Kv2.1 ectodomain-directed monoclonal IgG_{2a} antibody K39/25 was generated for these studies by standard procedures (Bekele-Arcuri et al., 1996) from mice immunized with a synthetic peptide corresponding to the S1-S2 loop of Kv2.1 (a.a. 207–225, CLPELQSL DEFGQSRDNPQL). Staining of surface Kv2.1 was performed on fixed neurons in the absence of detergent permeabilization (Shi et al., 1996). Neurons were then permeabilized and stained with the second (internal) primary antibody and secondary antibody as described above.

Microscopy and Quantification

Fluorescent images of cells were captured into Adobe Photoshop from a SPOT cooled CCD 24 bit color digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Zeiss Axioskop 2 microscope (Oberkochen, Germany) with a 100×, 1.4 numerical aperture (NA) or a 40×, 1.3 NA lens, using the software supplied with the SPOT camera. For quantitation of staining intensity presented in Figures 1 and 2, NIH image software was used to analyze raw data. Intensity analysis was used to graphically illustrate the degree and magnitude of the clustering of membrane proteins.

Acknowledgments

We thank Drs. Neta Dean, JoAnne Engebrecht, and Matthew Rasband for critically reading this manuscript. This work was supported by a grant from the National Institutes of Health (NS34375) to J. S. T.

Received November 11, 1999; revised December 9, 1999.

References

Banker, G.A., and Cowan, W.M. (1977). Rat hippocampal neurons in dispersed cell culture. Brain Res. *126*, 397–425.

Baranauskas, G., Tkatch, T., and Surmeier, D.J. (1999). Delayed rectifier currents in rat globus pallidus neurons are attributable to Kv2.1 and Kv3.1/3.2 K(+) channels. J. Neurosci. *19*, 6394–6404.

Bekele-Arcuri, Z., Matos, M.F., Manganas, L., Strassle, B.W., Monaghan, M.M., Rhodes, K.J., and Trimmer, J.S. (1996). Generation and characterization of subtype-specific monoclonal antibodies to K+ channel α- and β-subunit polypeptides. Neuropharmacology *35*, 851–865.

Brenman, J.E., Topinka, J.R., Cooper, E.C., McGee, A.W., Rosen, J., Milroy, T., Ralston, H.J., and Bredt, D.S. (1998). Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1A. J. Neurosci. *18*, 8805–8813.

Chandy, K.G., and Gutman, G.A. (1995). Voltage-gated potassium

channel genes. In Ligand- and Voltage-Gated Ion Channels, R.A. North, ed. (Boca Raton, FL: CRC Press), pp. 1–71.

Dargemont, C., Le Bivic, A., Rothenberger, S., Iacopetta, B., and Kuhn, L.C. (1993). The internalization signal and the phosphorylation site of transferrin receptor are distinct from the main basolateral sorting information. EMBO J. *12*, 1713–1721.

de Hoop, M., von Poser, C., Lange, C., Ikonen, E., Hunziker, W., and Dotti, C.G. (1995). Intracellular routing of wild-type and mutated polymeric immunoglobulin receptor in hippocampal neurons in culture. J. Cell Biol. *130*, 1447–1459.

Dotti, C.G., and Simons, K. (1990). Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. Cell *62*, 63–72.

Du, J., Tao-Chang, J.-H., Zerfas, P., and McBain, C.J. (1998). The K^+ channel, Kv2.1, is apposed to astrocytic processes and is associated with inhibitory postsynaptic membranes in hippocampal and cortical principal neurons and inhibitory interneurons. Neuroscience *84*, 37–48.

Epperson, A., Bonner, H.P., Ward, S.M., Hatton, W.J., Bradley, K.K., Bradley, M.E., Trimmer, J.S., and Horowitz, B. (1999). Molecular diversity of Kv α and β subunits expression in canine gastrointestinal smooth muscles. Am. J. Physiol. *277*, G127–G136.

Garaschuk, O., Yaari, Y., and Konnerth, A. (1997). Release and sequestration of calcium by ryanodine-sensitive stores in rat hippocampal neurones. J. Physiol. *502*, 13–30.

Goslin, K., and Banker, G.A. (1991). Rat hippocampal neurons in low-density culture. In Culturing Nerve Cells, K. Goslin and G.A. Banker, eds. (Cambridge, MA: MIT Press), pp. 251–281.

Hata, Y., Nakanishi, H., and Takai, Y. (1998). Synaptic PDZ domain-containing proteins. Neurosci. Res. *32*, 1–7.

Hell, J.W., Westenbroek, R.E., Warner, C., Ahlijanian, M.K., Prystay, W., Gilbert, M.M., Snutch, T.P., and Catterall, W.A. (1993). Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J. Cell Biol. *123*, 949–962.

Hille, B. (1992). Ionic Channels of Excitable Membranes (Sunderland, MA: Sinauer).

Hoffman, D.A., Magee, J.C., Colbert, C.M., and Johnston, D. (1997). K^+ channel regulation of signal propagation in dendrites of hippocampal pyramidal cells. Nature *387*, 869–875.

Humphrey, J.S., Peters, P.J., Yuan, L.C., and Bonifacino, J.S. (1993). Localization of TGN38 to the trans-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. J. Cell Biol. *120*, 1123–1135.

Hwang, P.M., Fotuhi, M., Bredt, D.S., Cunningham, A.M., and Snyder, S.H. (1993). Contrasting immunohistochemical localizations in rat brain of two novel K^+ channels of the *Shab* subfamily. J. Neurosci. *13*, 1569–1576.

Jareb, M., and Banker, G. (1998). The polarized sorting of membrane proteins expressed in cultured hippocampal neurons using viral vectors. Neuron *20*, 855–867.

Keller, P., and Simons, K. (1997). Post-Golgi biosynthetic trafficking. J. Cell Sci. *110*, 3001–3009.

Kim, J.H., and Huganir, R.L. (1999). Organization and regulation of proteins at synapses. Curr. Opin. Cell Biol. *11*, 248–254.

Kim, E., Niethammer, M., Rothschild, A., Jan, Y.N., and Sheng, M. (1995). Clustering of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases. Nature *378*, 85–88.

Korkotian, E., and Segal, M. (1997). Calcium-containing organelles display unique reactivity to chemical stimulation in cultured hippocampal neurons. J. Neurosci. *17*, 1670–1682.

Laube, G., Roper, J., Pitt, J.C., Sewing, S., Kistner, U., Garner, C.C., Pongs, O., and Veh, R.W. (1996). Ultrastructural localization of Shaker-related potassium channel subunits and synapse-associated protein 90 to septate-like junctions in rat cerebellar Pinceaux. Brain Res. Mol. Brain Res. *42*, 51–61.

MacKinnon, R. (1991). Determination of the subunit stoichiometry of a voltage-activated potassium channel. Nature *350*, 232–235.

Maletic-Savatic, M., Lenn, N.J., and Trimmer, J.S. (1995). Differential spatiotemporal expression of K+ channel polypeptides in rat hippocampal neurons developing in situ and in vitro. J. Neurosci. *15*, 3840–3851.

Muller, B.M., Kistner, U., Veh, R.W., Cases-Langhoff, C., Becker, B., Gundelfinger, E.D., and Garner, C.C. (1995). Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the Drosophila discs-large tumor suppressor protein. J. Neurosci. *15*, 2354–2366.

Muller, B.M., Kistner, U., Kindler, S., Chung, W.J., Kuhlendahl, S., Fenster, S.D., Lau, L.F., Veh, R.W., Huganir, R.L., Gundelfinger, E.D., and Garner, C.C. (1996). SAP102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. Neuron *17*, 255–265.

Murakoshi, H., and Trimmer, J.S. (1999). Identification of the Kv2.1 K+ channel as a major component of the delayed rectifier K+ current in rat hippocampal neurons. J. Neurosci. *19*, 1728–1735.

Murakoshi, H., Shi, G., Scannevin, R.H., and Trimmer, J.S. (1997). Phosphorylation of the Kv2.1 K+ channel alters voltage-dependent activation. Mol. Pharmacol. *52*, 821–828.

Nakahira, K., Shi, G., Rhodes, K.J., and Trimmer, J.S. (1996). Selective interaction of voltage-gated K+ channel beta-subunits with alpha-subunits. J. Biol. Chem. *271*, 7084–7089.

Neame, S.J., and Isacke, C.M. (1993). The cytoplasmic tail of CD44 is required for basolateral localization in epithelial MDCK cells but does not mediate association with the detergent-insoluble cytoskeleton of fibroblasts. J. Cell Biol. *121*, 1299–1310.

Pongs, O. (1999). Voltage-gated potassium channels: from hyperexcitability to excitement. FEBS Lett. *452*, 31–35.

Rhodes, K.J., Keilbaugh, S.A., Barrezueta, N.X., Lopez, K.L., and Trimmer, J.S. (1995). Association and colocalization of K+ channel α- and β-subunit polypeptides in rat brain. J. Neurosci. *15*, 5360–5371.

Rhodes, K.J., Strassle, B.W., Monaghan, M.M., Bekele-Arcuri, Z., Matos, M.F., and Trimmer, J.S. (1997). Association and colocalization of Kvb1.1 and Kvb2 with Kv1 α subunits in mammalian brain K+ channel complexes. J. Neurosci. *17*, 8246–8258.

Scannevin, R.H., Murakoshi, H., Rhodes, K.J., and Trimmer, J.S. (1996). Identification of a cytoplasmic domain important in the polarized expression and clustering of the Kv2.1 K+ channel. J. Cell Biol. *135*, 1619–1632.

Shamotienko, O.G., Parcej, D.N., and Dolly, J.O. (1997). Subunit combinations defined for K+ channel Kv1 subtypes in synaptic membranes from bovine brain. Biochemistry *36*, 8195–8201.

Sharma, N., D'Arcangelo, G., Kleinklaus, A., Halegoua, S., and Trimmer, J.S. (1993). Nerve growth factor regulates the abundance and distribution of K^+ channels in PC12 cells. J. Cell Biol. *123*, 1835–1843.

Sheng, M., and Pak, D.T. (1999). Glutamate receptor anchoring proteins and the molecular organization of excitatory synapses. Ann. NY Acad. Sci. *868*, 483–493.

Shi, G., Kleinklaus, A.K., Marrion, N.V., and Trimmer, J.S. (1994). Properties of Kv2.1 K+ channels expressed in transfected mammalian cells. J. Biol. Chem. *269*, 23204–23211.

Shi, G., Nakahira, K., Hammond, S., Rhodes, K.J., Schechter, L.E., and Trimmer, J.S. (1996). Beta subunits promote K+ channel surface expression through effects early in biosynthesis. Neuron *16*, 843–852.

Songyang, Z., Fanning, A.S., Fu, C., Xu, J., Marfatia, S.M., Chishti, A.H., Crompton, A., Chan, A.C., Anderson, J.M., and Cantley, L.C. (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science *275*, 73–77.

Stowell, J.N., and Craig, A.M. (1999). Axon/dendrite targeting of metabotropic glutamate receptors by their cytoplasmic carboxy-terminal domains. Neuron *22*, 525–536.

Stuart, G., Spruston, N., Sakmann, B., and Hausser, M. (1997). Action potential initiation and backpropagation in neurons of the mammalian CNS. Trends Neurosci. *20*, 125–131.

Swanson, R., Marshall, J., Smith, J.S., Williams, J.B., Boyle, M.B., Folander, K., Luneau, C.J., Antanavage, J., Oliva, C., Buhrow, S.A., et al. (1990). Cloning and expression of cDNA and genomic clones

encoding three delayed rectifier potassium channels in rat brain. Neuron *4*, 929–939.

Tejedor, F.J., Bokhari, A., Rogero, O., Gorczyca, M., Zhang, J., Kim, E., Sheng, M., and Budnik, V. (1997). Essential role for dlg in synaptic clustering of Shaker K+ channels in vivo. J. Neurosci. *17*, 152–159.

Thomas, U., Kim, E., Kuhlendahl, S., Koh, Y.H., Gundelfinger, E.D., Sheng, M., Garner, C.C., and Budnik, V. (1997). Synaptic clustering of the cell adhesion molecule Fasciclin II by Discs-large and its role in the regulation of presynaptic structure. Neuron *19*, 787–799.

Trimmer, J.S. (1991). Immunological identification and characterization of a delayed rectifier K+ channel polypeptide in rat brain. Proc. Natl. Acad. Sci. USA *88*, 10764–10768.

Trimmer, J.S. (1998). Regulation of ion channel expression by cytoplasmic subunits. Curr. Opin. Neurobiol. *8*, 370–374.

Trimmer, J.S. (1999). Sorting out receptor trafficking. Neuron *22*, 411–412.

VanDongen, A.M., Frech, G.C., Drewe, J.A., Joho, R.H., and Brown, A.M. (1990). Alteration and restoration of K+ channel function by deletions at the N- and C-termini. Neuron *5*, 433–443.

West, A.E., Neve, R.L., and Buckley, K.M. (1997). Identification of a somatodendritic targeting signal in the cytoplasmic domain of the transferrin receptor. J. Neurosci. *17*, 6038–6047.

Zito, K., Fetter, R.D., Goodman, C.S., and Isacoff, E.Y. (1997). Synaptic clustering of Fascilin II and Shaker: essential targeting sequences and role of Dlg. Neuron *19*, 1007–1016.